(12) INTERNATIONAL APPLICATION PUBLISHED UNDER THE PATENT COOPERATION TREATY (PCT)

(19) World Intellectual Property Organization
International Bureau

(43) International Publication Date
18 September 2003 (18.09.2003)

PCT

(10) International Publication Number
WO 03/075903 A2

(51) International Patent Classification⁷: A61K 31/00

(21) International Application Number: PCT/NL03/00174

(22) International Filing Date: 10 March 2003 (10.03.2003)

(25) Filing Language: English

(26) Publication Language: English

(30) Priority Data:
02075964.3 8 March 2002 (08.03.2002) EP
60/372,962 16 April 2002 (16.04.2002) US

(71) Applicant *(for all designated States except US)*: UNIVERSITEIT LEIDEN [NL/NL]; Stationsweg 46, NL-2312 AV Leiden (NL).

(72) Inventors; and

(75) Inventors/Applicants *(for US only)*: ALIA, . [IN/NL]; Jo Vincentlaan 14, NL-2343 PK Oegstgeest (NL). BACKENDORF, Claude, Maria, Pierre [LU/NL]; Boerhavelaan 307, NL-2334 EM Leiden (NL). Matysik, Jörg [DE/NL]; Jo Vincentlaan 14, NL-2343 PK Oegstgeest (NL).

(74) Agent: PRINS, A.W.; Nieuwe Parklaan 97, NL-2587 BN Den Haag (NL).

(81) Designated States *(national)*: AE, AG, AL, AM, AT (utility model), AT, AU, AZ, BA, BB, BG, BR, BY, BZ, CA, CH, CN, CO, CR, CU, CZ (utility model), CZ, DE (utility model), DE, DK (utility model), DK, DM, DZ, EC, EE (utility model), EE, ES, FI (utility model), FI, GB, GD, GE, GH, GM, HR, HU, ID, IL, IN, IS, JP, KE, KG, KP, KR, KZ, LC, LK, LR, LS, LT, LU, LV, MA, MD, MG, MK, MN, MW, MX, MZ, NI, NO, NZ, OM, PH, PL, PT, RO, RU, SC, SD, SE, SG, SK (utility model), SK, SL, TJ, TM, TN, TR, TT, TZ, UA, UG, US, UZ, VC, VN, YU, ZA, ZM, ZW.

(84) Designated States *(regional)*: ARIPO patent (GH, GM, KE, LS, MW, MZ, SD, SL, SZ, TZ, UG, ZM, ZW), Eurasian patent (AM, AZ, BY, KG, KZ, MD, RU, TJ, TM), European patent (AT, BE, BG, CH, CY, CZ, DE, DK, EE, ES, FI, FR, GB, GR, HU, IE, IT, LU, MC, NL, PT, RO, SE, SI, SK, TR), OAPI patent (BF, BJ, CF, CG, CI, CM, GA, GN, GQ, GW, ML, MR, NE, SN, TD, TG).

Published:

— *without international search report and to be republished upon receipt of that report*

For two-letter codes and other abbreviations, refer to the "Guidance Notes on Codes and Abbreviations" appearing at the beginning of each regular issue of the PCT Gazette.

WO 03/075903 A2

(54) Title: USES OF PROLINE AND FUNCTIONAL EQUIVALENTS THEREOF AND COMPOSITIONS CONTAINING SAID COMPOUNDS

(57) Abstract: The invention relates to the fields of human medicine, cosmetics and food, more particular to the prevention or inhibition of oxidation by quenching of reactive oxygen species and/or radicals. The use of proline and/or a functional equivalent thereof, is claimed for the protection against damage by ROS and/or radicals. Pharmaceutical, topical, and neutraceutical compositions and methods to protect cells and tissues against damage by ROS and/or radicals are also claimed. Diagnostic methods are claimed for the detection of ROS or radicals induced disease.

Title: Uses of proline and functional equivalents thereof and compositions containing said compounds.

The invention relates to the fields of human medicine, cosmetics and food, more particular to the prevention or inhibition of oxidation by quenching of reactive oxygen species.

There is now accumulating evidence that reactive oxygen species play a major role in the aetiology of a large number of human diseases. Activated oxygen is a major component in the toxicity of many drugs and has been directly linked with aging and carcinogenesis. Natural anti-oxidants, such as proline and functional equivalents thereof, can thus contribute significantly to the general well-being of humans and animals.

In plants, less than 5% of the total pool of free amino acids is provided by proline. However, under various forms of stress, the concentration increases up to 80% of the amino acid pool. This observation indicates that a high proline concentration is favourable for plants under stress conditions. In addition to the role of proline as osmolyte and reservoir of carbon and nitrogen etc, proline has been shown to protect plants against free radical induced damage. Recently, we disclosed that, in a certain pathway in thylakoid membranes, proline seems to play a role in quenching of singlet oxygen, erroneously produced in said pathway (1).

Herein we disclose that reactive oxygen species (ROS) and other radicals quenching by proline, or a functional equivalent thereof, can be used to e.g. prevent or diminish possible damage to a wide variety of substances, tissues and cells.

It was found that ROS quenching by proline is far more widespread than the action, disclosed by us, in the thylakoid membranes of certain plants.

We herein disclose the mechanism of action of quenching ROS and other radicals by proline or a functional equivalent thereof and the use of this quenching mechanism in many different fields, especially those related to human and animal health..

In this application, we disclose the use of proline and/or a functional equivalent thereof, and/or protein and/or peptide rich in said proline and/or a functional equivalent thereof for quenching radicals, in particular reactive oxygen species (ROS). Said functional proline equivalent has at least in kind, if not in amount the same function of quenching radicals as proline, said quenching comprising physical and/or chemical quenching of radicals, in particular of ROS and/or radicals. Any derivative of proline, with the same quenching ability in kind if not in amount, is also comprised in the invention

Quenching ROS and/or radicals can be useful in many applications. Chemical reactions that depend on radical initiation can be stopped. Side reactions involving radicals can be inhibited, etc.

Particularly, when radicals are unwanted, quenching according to the present invention is useful. A lot of materials, in particular biological materials, can suffer damage from ROS, which includes radical and non-radical species, such as for example superoxide, nitric oxide, hydroxyl radical, alkoxyl radical, lipid peroxyl radical, peroxynitrite, and hydrogen peroxide etc, and other radicals. ROS and/or radical induced damage plays a role in pathologies as for example damaging radiation induced skin damage, fibrosis inflammatory skin diseases, skin cancer, psoriasis, periodontal disease, pathological conditions of the stomach and gut affecting mucosal barrier function, pathologies of lung, oral and nasal epithelium due to cigarette smoke, photo-aging of the skin, wound healing, aging and age related diseases, cardiovascular diseases, artherosclerosis, Alzheimer's disease and neurodegenerative diseases, dementia, Parkinson's disease,

inflammatory airway disorders, male infertility, and breast cancer. Because proline is safe for biological materials, and in general for quenching of ROS and/or radicals, it is an excellent and safe method for preventing or diminishing damage caused by ROS and/or radicals. Of course, equivalents, which retain the quenching function of proline (functional equivalents) such as hydroxyproline, can also be used. Also, precursor molecules, that enable the production of proline, for instance in cells, are comprised in the term equivalent. Hydroxyproline is a preferred functional equivalent.

Because proline or a functional equivalent thereof is also functional when incorporated in a protein or a peptide or an equivalent thereof, this application also discloses proteins and peptides containing proline. Said proline rich molecules contain preferably at least 8 mol% proline, even more preferably at least 10 mol% , even more preferably 16 mol %. Of course, when the proline content of a protein is not high, more protein can be administered to give the same result. There is a correlation between the amount of proline present in a protein and the quenching effect for ROS. Because proline is not toxic for mammals, said proline rich protein may even comprise up to 35 mol% of proline or functional equivalent or even more preferably 40 mol% or even higher. Said proline rich proteins are, for example, but not limited to the gene family of proline-rich proteins (PrP's) (17) and/or small proline-rich proteins (SPRR)(14) and/or Late Envelope Proteins (LEP)(18), and/or collagen, and/or elastin, and/or prolamine, and/or casein, and/or proline-rich muscle proteins and/or poly-proline peptides for preventing and/or diminishing damage by ROS and/or radicals. Said LEP proteins (late envelope protein), are present in the skin and have an N-terminal domain (rich in proline) which is very similar to the corresponding domain in SPRR proteins (most probably because they have a common ancestor). Said proline rich proteins (PrPs) are present in e.g. saliva and tears. The proline repeats in these proteins are different from those in SPRR and LEP, but these proteins a comparable

antioxidant function as SPRR. All of abovementioned proteins may be the source of proline of the invention.

Thus, in one embodiment, said proline and/or a functional equivalent thereof, can be used for quenching ROS and/or radicals. With proline amino acid and/or an equivalent thereof, the minimum amount of proline which at least in part exhibits quenching is a composition comprising at least 0.01 millimolar proline and/or a functional equivalent thereof. More preferably said amount of proline is at least 1 millimolar, even more preferably said amount of proline is 10 millimolar. Protein and/or peptide rich in said proline and/or a functional equivalent thereof can also be used for quenching ROS and/or radicals. Therefore, this patent application also teaches the use of proline for quenching radicals, wherein at least part of the proline is provided as protein and/or peptide. Preferably, said protein and/or peptide comprises at least 8 mol% proline and/or a functional equivalent thereof. Preferably said protein or peptide is present in an amount of 0.01 micromolar. Even more preferably, said protein or peptide is present in an amount of 0.1 micromolar. Consequently, the present application also provides the use of proline or a functional equivalent thereof to prevent and/or diminish damage by ROS and/or radicals. Proline and/or functional equivalents can at least in part prevent damage to cells and tissues.

Proline and/or functional equivalent thereof can also be used for the preparation of a medicament, preferably to diminish or prevent damage to cells and/or tissues by ROS and/or radicals.

In another embodiment, said proline and/or functional equivalent thereof, which is able to quench ROS and/or radicals, can be added to a food substance, or to a topical composition, or to a pharmaceutical composition. In food, said proline and/or functional equivalent thereof can diminish or prevent damage by ROS and/or radicals to the food during storage and preparation, and also to the consumer of the food by preventing or diminishing damage by ROS and/or radicals, for instance to the mucosa of the digestive tract.

In another embodiment, the invention provides a topical composition comprising proline and/or a functional equivalent thereof, and a suitable carrier or diluent. In a pharmaceutical composition, said proline and/or a functional equivalent can diminish or prevent damage by ROS and/or radicals to the pharmaceutical composition during storage and preparation, and, also to the consumer of the pharmaceutical composition by preventing or diminishing damage by ROS and/or radicals to the cells and tissues of the body. Proline is a common substance and is occasionally present in medicaments, pharmaceutical products and food substances. Up to now, proline has not been added as an active ROS and/or radical quenching molecule. Therefore, the invention also provides a composition comprising proline and/or a functional equivalent thereof characterized in that proline or a functional equivalent is an active substance in the preparation of a pharmaceutical composition. An active substance of a pharmaceutical composition is a substance which performs the specific action of the pharmaceutical composition.

Abovementioned topical composition is for example a sun-screening or sun-blocking product and can help to avoid or diminish photo-induced damage of the skin. Therefore, the invention also provides a method to prevent damage to the skin during sustained tanning or after tanning comprising applying said composition prior to exposure of the body to a ROS and/or radicals inducing condition, for example damaging radiation, including but not limited to ionising or cosmic radiation, solar radiation or artificial radiation from for example sun-tanning devices. Said topical composition may also be for example an after sun product which is applied to at least that part of the skin which has been exposed tot the sun or another source of UV rays. Because the initial ROS and/or radical damage starts a chain of events in the skin and the tissues of a subject, said chain of events may be interfered with by the application of the topical composition and proline may quench the ROS and/or radicals that are formed later in the tissue. The proline in said topical composition is effective at least at a concentration of 0.01 millimolar, preferably at a

concentration of 0.05 millimolar, even more preferably at a concentration of 0.1 millimolar or higher. In the event that proline in said topical composition is incorporated in proteins or peptides, the proteins or peptide have to comprise at least 8 mol% of proline and have to be applied in a concentration of 0.01 micromolar, more preferably 0.1 micromolar

This application shows the ROS and/or radical quenching ability of feeding proline to mice. With proline as the active ingredient or substance for quenching ROS and/or radicals, sun-blocking or after sun care for the skin may also be performed by oral intake of the proline or its functional equivalent. The proline reaches the skin by the circulation and this effects in proline accumulation in the skin. A reaction to radiation can be effectively inhibited by using oral preparations of the invention. Therefore, this application also teaches an oral sun-tanning product comprising a radical quenching substance. Said oral sun-tanning product thus comprises a ROS and/or radical quenching substance, which accumulates in skin. Said oral sun-tanning product preferably comprises as an active substance proline and/or a functional equivalent thereof. Additionally, the invention teaches an oral after-sun product comprising a ROS and/or radical quenching substance, which preferably accumulates in skin. Said oral sun-tanning product preferably comprises as an active substance proline and/or a functional equivalent thereof.

Preferred topical compositions also comprise cosmetic products like: soap, shampoo, hair dye, hair-bleaching solutions, nail-care products, and dental-care products. Therefore, said invention also provides a method to protect hair, eyes, nails, skin and teeth against damage by ROS and/or radicals comprising applying said composition prior to exposure of the body to a ROS and/or radical inducing condition, for example staining, bleaching, cleaning, or radiation. The same effect may also be reached by applying the cosmetic products after the ROS and/or radical inducing conditions, like for example the application of a proline providing topical cosmetic product to hair after bleaching or staining. The present invention also teaches that the cornified envelope of corneocytes in the outermost

layer of human skin, has an intrinsic ROS and/or radical quenching ability, due to the presence of SPRR proteins, whose expression is dramatically increased after long-term exposure to UV light and in aged skin.

This application also teaches the results of feeding proline to knockout mice that show an increased sensitivity to ROS and/or radicals, and have a short life expectancy, due to numerous tissue failures. Feeding proline could slow this process down in homozygous mice resulting in a higher frequency of young mice being born alive and also staying alive from mothers that were kept on a high-proline diet. Therefore, this application also teaches the use of a composition comprising proline as an active substance for at least in part preventing ageing and/or decay of the treated tissue. As a direct causative link between ROS and/or radicals and aging has been made in humans, this application teaches also the use of a composition, containing as an active substance proline or functional equivalents thereof, as an anti-ageing substance. Besides, this invention teaches the use of said proline composition as a protective agent for humans exposed to increased levels of ionizing radiation (e.g. airline- and fighter-jet pilots, cosmonauts, astronauts, workers in nuclear power plants, radiologists). This composition can be taken orally or can be applied to some part of the body. In yet another embodiment, the invention provides a pharmaceutical composition comprising proline and/or a functional equivalent thereof as an active substance- and a suitable carrier or diluent.

The invention also teaches the use of proline and/or a functional derivative thereof for the preparation of a medicament for the treatment of ROS-related disease comprising damaging radiation induced skin damage, inflammatory skin diseases, fibrosis, skin cancer, psoriasis, periodontal disease, pathological conditions of the stomach and gut affecting mucosal barrier function, pathologies of lung, oral and nasal epithelium due to cigarette smoke, photo-aging of the skin, wound healing, age related

diseases, cardiovascular diseases, artherosclerosis, Alzheimer's disease and neurodegenerative diseases, dementia, Parkinson's disease, inflammatory airway disorders, male infertility, and/or breast cancer.

The invention also provides a method to protect cells and tissues of a mammal against damage by ROS and/or other radicals by administration of said pharmaceutical composition prior to, during and/or after a ROS and/or radicals inducing treatment or diagnosis.

Food with high proline contents deposits the proline and/ or proline-rich proteins or –peptides in the digestive tract. In said digestive tract,, said proline and or proline-rich proteins or –peptides quench topical ROS and/or radicals and prevent damage of the digestive tract cells and tissues. In this way, food intake with additional proline or proline-rich proteins or -peptides may prevent and/ or alleviate stomach ulcers, duodenal ulcers, and lower gut inflammation and/or chronic inflammatory reactions such as for example, but not limited to Crohn's disease. Therefore, the invention teaches the use of proline and/or a functional equivalent thereof, for the preparation of a food anti-oxidant and/or nutraceutical composition. In another embodiment, the invention provides a food anti-oxidant and/or nutraceutical composition comprising proline and/or a functional equivalent thereof.

The invention also teaches a method to protect the upper digestive tract (oral cavity, oesophagus), stomach and intestines against oxidative damage comprising oral administration of food comprising said food anti-oxidant and/or neutraceutical. A higher intake of said proline and/or a functional equivalent thereof may lead to a better protection against damage by ROS and/or radicals in the tissues of the body.

Proline and proline rich proteins or peptides are water soluble, therefore, a medicament comprising proline or a functional equivalent thereof to protect cells and tissues against ROS and/or radicals can also be administered intravenously or subcutaneously, or by inhalation to individuals or animals, prior to treatment with ROS inducing methods, like for example, but not limited to: irradiation in cancer treatment,

chemotherapy, or photodynamic therapy. The non-toxic nature and the pleasant taste of proline makes it very suitable to mix in the daily diet of humans and animals thereby protecting said organisms from oxidative damage. Therefore the present invention also teaches a method to protect humans and animals against oxidative damage comprising increasing the proline level of their food and drink to at least 0.01 millimolar by adding proline and/or a functional equivalent.

The induction of or increase in proline levels (mainly in the form of, but not limited to, SPRR proteins) in the skin, neurons, lungs, oesophagus, stomach and gut as shown in the present invention, after various stress inducing conditions, enables a new way of diagnosing the health status of tissues and cells. Therefore the present application also teaches a method to diagnose ROS- or radical-related damage and/or disease or the sensitivity for such diseases comprising measuring the level of proline or a functional equivalent thereof and/or measuring the expression of proline-rich protein genes (such as, but not limited to, SPRR, PrP, LEP gene families) in a sample tissue, in particular in a sample of damaged or diseased tissue.

Detailed description

The dramatic increase of proline occurs over several hours or days in plants after application of stress. The accumulation and protective effect of proline has been observed in many higher plants and bacteria as well as in protozoa, algae, marine invertebrates and insects (2). Stresses such as cold, heat, salt, drought, UV or heavy metal stress cause a significant increase of the proline concentration in a variety of plants. Interpretations of proline accumulation vary from its role as a useful adaptive response, helping organisms to withstand the effect of stress, to merely a consequence of stress induced damage to the cells (3). Transgenic plants, which are not able to produce proline, have a significantly lower stress tolerance (4).

Stress is the result of the sum of damages in all cellular components (lipids, proteins and nucleic acids). All stresses induce the production of reactive oxygen species, especially singlet oxygen and free radicals that are known to break DNA, impair the function of proteins and are responsible for lipid peroxidation. Plants have evolved diverse strategies of acclimatisation and avoidance to cope with adverse environmental conditions. These strategies include accumulation of compatible solutes like for example glycinebetaine, proline, and mannitol.

Reactive oxygen species cause stress to biological systems. Stress induces in turn production of reactive oxygen species. Therefore, a mechanism to interrupt such an autocatalytic process is required. Under normal circumstances, concentrations of oxygen radicals remain low because of the activity of protective enzymes including superoxide dismutase, catalase and ascorbate peroxidase (6). Under stress, accumulation of compatible solutes occurs, in addition to the increase in the activities of detoxifying enzymes. Interestingly, among various compatible solutes, proline has been shown to protect plants against singlet oxygen, and free radical induced damages (7)

As mentioned before, proline accumulates in high amount in several plants under stress. This accumulation of proline can protect plants against damage by reactive oxygen species.

The name of proline has been derived from "pyrrolidine" by Emil Fischer in 1904. The amino acid L-proline (L-pyrrolidine 2-carboxy acid, $C_5H_9NO_2$, Fig. 1A) in its pure form is a colourless substance, highly soluble in water, well soluble (unlike all other amino acids) in alcohols, sparingly soluble in benzene and acetone and insoluble in ether. In aqueous solution at physiological pH the free amino acid proline occurs in its zwitterionic form, carrying negative charge on the deprotonated carboxyl group and positive charge on the doubly protonated amino function. Therefore it also can be referred to as "proline betaine". Due to ring puckering, two conformations of the pyrrolidine ring exist, called *up* and *down*.

Incorporated into peptides, the substituents of the pyrrolidine ring can also assume two conformations, *trans* and *cis*, depending on whether the peptide carbonyl oxygen atoms are pointing to each other (*cis*) or not (*trans*). Proline incorporation has a destabilizing effect on the secondary structure of proteins. As a rotationally hindered secondary amine (unique among all neutral amino acids), proline hardly matches the steric requirements needed for forming an α-helix. On the opposite, proline residues are often at the end of an α helix. In the formation of β turns, often proline and hydroxyproline ([2S]-[4R]-hydroxy pyrrolidine 2-carbonic acid, Fig. 1B) are involved. In general, β turn-rich proteins contain a high amount of (hydroxy)proline, e.g., casein (7 %) and prolamine (20 %). Collagen (26 %) forms a special triple helix.

Primary amino acids R-CH(COOH)(NH_2) react chemically as expected from a compound carrying an amino and a carboxyl group. They can be oxidised by thermal, chemical or enzymatic decarboxylation. The products are amides R-CO(NH_2), aldehydes R-CH-CHO $_{\gamma}$ and amines R-CH_2-NH_2. In case of the secondary amino acid proline, the amide is the cyclic 2-pyrrolidone.

In addition to the reactions similar to the primary amino acids, proline can reversibly perform a ring-opening reaction by addition of a molecule of water, forming glutamic acid γ-semialdehyde (Fig. 2), a equivalent of glutamate, which is the most central amino acid in the network of amino acid biosynthesis. Since the semi-aldehyde is reduced two electron equivalents higher as glutamate, proline can be considered as an "electron rich glutamate", and hence the redox system proline/glutamate provides a mitochondrial "electron sponge" (Fig. 3). In general, proline is formed from glutamate in plants. Alternatively, proline is also enzymatically synthesised from ornithine by exchange of the δ-amino to a γ-aldehyde group forming the glutamic acid semialdehyde as precursor of proline. Hydroxyproline is produced in the peptide-bound form from proline by oxidation with an ascorbic acid-dependent monooxygenase. Thus, glutamate, ornithine, glutamic acid semialdehyde

and hydroxyproline are suitable precursors of proline and thus preferred equivalents of proline.

The protective action of free proline against reactive oxygen species

"Normal" molecular oxygen ($^3O_2$) is in its electronic ground state, which is a triplet state. Due to spin-conservation rules, triplet oxygen is rather inactive to biological material. The electronically excited singlet oxygen ($^1O_2$) (Fig. 4) however is highly reactive and rapidly oxidises amino acids, lipids and DNA. Free radicals produced by reaction with $^3O_2$ react similarly aggressively. The one-electron reduced superoxide radical ($HO_2^\bullet$ or $O_2^{1-\bullet}$) and the hydroxyl radical ($OH^\bullet$), which is on the same redox level as the peroxide, are the main oxygen radical compounds (Fig. 5). Also the $NO^\bullet$ radical, found recently as active component in several biological systems, can cause unwanted damages. In the following, the chemical reactivity of proline with reactive oxygen species is discussed with the aim to understand the molecular mechanism of the protective effect of proline under stress in plants.

Proline quenches singlet oxygen

First some important properties of singlet oxygen will be introduced. Subsequently, the electronic state of singlet oxygen, its production (*in vitro* and in plants) and its chemical reactivity with proline and other amines are discussed.

Electronic state of singlet oxygen

Since chemical properties depend on the electronic structure, electronically excited molecules undergo different chemical reactions as the same molecules in their electronic ground state. Therefore, molecules in different electronic states have to be regarded as different chemical species. Most molecules have a singlet state (S) as electronic ground state, i.e., all electrons are paired and there is no electronic spin. There are,

however, important exceptions. One of them is molecular oxygen (O_2) which has triplet state (T) as electronic ground state. "Normal" molecular oxygen (Fig. 4), which is in the air we are breathing, is therefore in triplet state ($^3O_2$) (denoted as $^1\Sigma g^-$). Other examples for compounds with T-ground state are given by carbene compounds (CR_2). Due to quantum mechanical spin-conservation rules, chemical reactions between species in triplet and singlet states are spin forbidden, and therefore have a high kinetic reaction barrier. This is the reason why atmospheric oxygen does not react easily with organic matter, although such reaction would be energetically very favourable. In the electronically excited state (denoted as $^1\Delta g$), oxygen becomes an electronic singlet (Fig. 4). Reactions of singlet oxygen with organic molecules are not spin forbidden, and have much less activation energy. One way to circumvent the spin-dependent selection rules is provided by catalysis with transition metals (via spin-orbit coupling of their d-orbitals). Lifetime and action radius of singlet oxygen depend on the surrounding medium. In aqueous solution singlet oxygen has a lifetime of ca. 3 μs and can diffuse almost 0.2 μm. This is a rather long lifetime, as compared to other reactive oxygen species. Consequently, chemical reactions of singlet oxygen are much more specific as compared to $OH^{\bullet}$ radicals. Singlet oxygen reacting with DNA interacts selectively with guanine, since it has the lowest redox potential among the nucleotides constituting DNA.

Production of singlet oxygen

The usual way to produce singlet oxygen *in vitro* is by irradiation of photosensitisers. These are dyes with high quantum yield for triplet formation (Fig. 6, formulae [1] – [3]). In nature singlet oxygen is produced in the same photochemical way. For example P_{680}, the primary electron donor in reaction centres of photosystem II in plants, acts as photosensitiser and is the main source of singlet oxygen in plants. Another cellular source of singlet oxygen are heme proteins such as peroxidases. In plants under high light irradiation, the likelihood for the production of

singlet oxygen increases, because photons are absorbed faster than electrons are pumped. Singlet oxygen is directly involved in photobleaching of photosynthetic pigments, D1 protein degradation and protein cross-linking (8). Also various stress conditions produce singlet oxygen, which affect the structural integrity of the photosystem II membrane protein complex, and destabilise proteins and membranes further.

Chemical reactivity of singlet oxygen with proline

Our recent experiments (9) in an artificial system using toluidine blue as a sensitiser solution showed that proline is an excellent quencher for singlet oxygen in vitro (Fig. 8). Fig. 8a shows the production of singlet oxygen as detected by the formation of 2,2,6,6-tetramethylpiperidine-1-oxyl (TEMPO) by EPR spectrometry. Interestingly, the production of TEMPO was decreased in the presence of 5 and 10 mM proline (Fig. 8b & c) and completely abolished by the presence of proline at concentration as low as 20 mM (Fig. 8d). These results show that proline is a very effective singlet oxygen quencher. This is in line with the low ionisation potential (IP) of proline as discussed below. Glycine or different types of sugar have no effect on the singlet oxygen concentration. In the following paragraphs, the molecular mechanism of the chemical reaction of proline with singlet oxygen is discussed in detail.

The most characteristic feature of the chemical reactivity of singlet oxygen is its electrophilicity. Therefore, C=C or C=O double bonds as well as the lone pairs of sulphur and amines compounds are preferably the targets of singlet oxygen attack. Investigations of the reaction with amines have revealed that the kinetics of the reaction is controlled by the IP (i.e. the capability to provide an electron) of the amine. Therefore, the formation of a charge-transfer (CT) complex in a reversible reaction is assumed to be the initial step (Fig. 6, formula [4]).

An amine, having a low IP, is easier capable to form a CT complex, and can therefore quench singlet oxygen faster. IPs can be

determined either by photoelectron spectroscopy (PES) or electrochemically. The first electron removed from an amine can be assigned to the lone pair of the nitrogen atom. The value of the IP depends on the chemical structure of the amine. Due to the electron-donating character of alkyl substituents, tertiary amines have a lower IP than secondary amines, and secondary amines have a lower IP than primary amines. Therefore, charge transfer complexes of tertiary amines are more stable than secondary and secondary more than primary amines (Fig. 7). Also cyclic compounds are easier to ionise as open-chained amines. The larger the ring, the lower is the IP. The IP of enamines is lower than the IP of tertiary amines. IPs of peptide-bound amino acids are similar to that of isolated amino acids, since interactions among amino and carboxyl end groups are weak.

Pyrrolidine, which forms the 5-membered ring of proline, has a remarkable low IP of 8.0 eV. The substitution of the carboxyl group increases the IP slightly by withdrawing electrons from the ring. The decay of the initial CT complex of the amine with the singlet oxygen can occur either by physical (Fig. 6, formula [5]) or chemical (Formula [6]) quenching. For amines, both pathways are competing. Due to its low IP, proline forms CT complexes with singlet oxygen, and due to its structure, both, chemical and physical quenching is possible. Physical quenching works via a mechanism involving spin-orbit coupling restoring the original amine compound in its singlet ground state. For instance, azide (N_3^-) is known to quench via the physical pathway. In the case of chemical quenching, the structure of the amine is changed, and a reaction product is formed. One crucial aspect for the feasibility of a chemical pathway is the existence of α-hydrogen atoms and the possibility to form a $C{=}NH^+$ group. For highly constrained amines as 2,2,6,6-tetramethylpiperidine (TEMP), also another chemical pathway, producing stable nitroxide radicals, is possible. This chemical reaction is used for the detection of singlet oxygen by EPR spectroscopy (see above). Hence, the experimental

finding of the high capability of proline to quench singlet oxygen can be well understood by its chemical properties.

Reactivity of proline toward hydroxyl radicals

Compared to singlet oxygen, OH• radicals react much faster and with less selectivity. Both reactive species can react as strong oxidants, too, and are able to abstract hydrogen atoms. The involvement of hydroxyl OH• and superoxide O_2^{-}• radicals in oxidative stress is well known. The nitrosyl NO• radical is known to occur in biological systems and also can induce damages.

Formation of hydroxyl radicals

OH• radicals can be formed in several ways. Fenton's reaction for production of hydroxyl radicals by oxidation of Fe^{2+} ions is well known:

$$Fe^{2+} + H_2O_2 \rightarrow Fe^{3+} + OH\bullet + OH^{-} .$$

Also other metal ions as Cu^{1+} are able to reduce hydrogen peroxide (H_2O_2). In the presence of primary carbonate HCO_3^{-}, also Mn^{2+}, which occurs in high concentration in plants, can cause the disproportionation of hydrogen peroxide. Hydrogen peroxide also can form two OH• radicals either by heating or by photolyzation under UV irradiation. Organic peroxides as tertiary butyl hydroperoxide provide OH• radicals. Heme proteins undergoing oxygen-redox chemistry can release free radicals. Another method for the production of OH• radicals (together with H• radicals and solvated electrons e^{-}_{aq}) are γ-rays from a cobalt-60 source. Superoxide radicals are formed by the reaction of H• or e^{-}_{aq} with molecular oxygen

Reactions of hydroxyl radicals with proline

Smirnoff and Cumbes (10) have assessed the hydroxyl radical scavenging activity of various compatible solutes including proline which accumulate in plants under stress, and found that sorbitol, mannitol, myo-inositol and proline are effective hydroxyl radical scavengers. Proline

reacts with OH• under hydrogen abstraction by forming the most stable radical, which carries the spin on the C-5 atom since it is far from the carboxyl group and close to the nitrogen. In case of hydroxyproline, the radical with spin localisation on the C-4 is more stable.

Most of the OH• radicals generated *in vivo*, except during excessive exposure to ionising radiation, come from Fenton's reaction by oxidation of metals. Whether this reaction occurs and OH• radicals are released, depends on the state of complexation of the redox-active metal. Normally, metal ions in aqueous solution do not exist "naked" but are either hydrated or ligated by other molecules such as hydrogen carbonate or chelating compounds as EDTA, which have a strong effect on the chemical reactivity of the metal. Oxygen rapidly oxidises $Fe(OH)_2$ to Fe(III). Because at physiological pH, Fe(III) exists largely as insoluble polymeric $Fe(OH)_3$, sub-stoichiometrical amounts of chelating compounds can keep a trace of iron soluble, catalysing Fenton's reaction. Under these conditions, the reaction may occur in the complexation shell of the redox-active metal and may only affect the ligands. Such a "caged reaction" can effectively protect the surrounding biological material since the OH• radical cannot attack outside the cage. This means that binding of proline to redox-active metal ions can protect the surrounding biological tissue from damage by OH• radicals.

Proline residues in proteins

Singlet oxygen quenching

Proline incorporated into a peptide backbone becomes a tertiary amide. The IP of peptide-bound proline residues is similar to free proline, and therefore its capability to quench singlet oxygen is also similar. It has been shown, that proline-rich proteins, stabilising cell walls, are secreted by salt-adapted bean cells. Also in animal and human cells, production of proline-rich proteins has been described. Examples are the salivary proline-rich proteins (PrP's)(11) and the epithelial small proline-rich

proteins (SPRR's)(12). SPRR's are evolutionary related to late envelope proteins (LEP's) with which they share a conserved proline-rich domain. The three classes of proteins can be crosslinked by transglutaminases. PrP's are part of the salivary pellicle covering the tooth surface, whereas SPRR's (also named SPR's, cornifins or pancornulins) and LEP's are precursor proteins of the cornified cell envelope of the epidermal skin and internal squamous epithelia (lining of oral cavity, oesophagus, cervix and vaginal epithelium)(13). Interestingly, the expression of SPRR's is strongly induced after exposure of epidermal keratinocytes (14) or skin (15) to UV radiation.

Recently, it was shown that SPRR proteins are also strongly induced in axotomized neurons and play a role during nerve regeneration. We foresee a possible role of SPRR's as anti-oxidants in this process, where degeneration of the distal portion of the nerve is tightly linked with massive cell growth at the proximal site. As a matter of fact, the brain is especially sensitive to oxidative damage because of its high content of readily oxidized fatty acids and high use of oxygen.

A similar explanation might also hold for other internal organs and tissues where SPRR expression was found to be induced after various forms of stress. These include airway epithelium and the lining of the oesophagus, stomach and gut.

Hydroxyl radical quenching

Attack by OH• and HO_2• radicals is known to destabilise proteins. The peptide backbone can be cleaved by forming a carbon centred radical after α-hydrogen abstraction. Furthermore, side chains can be attacked. Proline residues can be preferred sites of radical attack. Proline can be oxidised to various compounds (Fig. 8). Formation of 4-hydroxyproline will not cause a cleavage of the polypeptide. Also the

formation of 5-hydroxyproline, which can hydrolyse to glutamate γ-semialdehyde, does not damage the amino-acid backbone. The observation of presence of γ-aminobutyric acid, glutamic acid, and 2-pyrrolidone, however, has to be explained by backbone cleavage. Oxidation of proline to 2-pyrrolidone provides a unique mechanism for peptide backbone cleavage that is not associated with the formation of a reactive carbonyl group. The very high content of proline and hydroxyproline in collagen is not always advantageous for the maintenance of tissue structures under metabolic conditions. Collagen degradation is a hallmark of photodamaged connective tissue. X-ray irradiation, applied in lung cancer, is known to induce fibrosis, i.e. an excessive accumulation of collagen, limiting the irradiation level in radiation therapy of thoracic tumours.

Caged reactions in the enzyme

If the redox-active metal is bound to the protein, the oxidation can be site specific. The Fenton-type generation of the OH• radical, is followed by the formation of a more stable carbon-centred radical. Similar as guanine becomes selectively oxidised within nucleic acids, "hole hopping" may also occur among amino acids, forming the most stable amino-acid radical. Such "caged" enzymatic reactions can be important for the function of an enzyme, and several proteins with oxidative modified amino acids in the reaction centre are known. Cross-linked amino acid residues are known (e.g. the reactive centres of cytochrome-*c* oxidase and galactose oxidase. In both cases, the two covalently bound amino acids are functionally important and are in direct neighbourhood of the metal. Site-directed oxidation of proline, histidine, arginine, lysine, threonine, tyrosine and cysteine has been reported (16). Hence, proline residues, which are very good singlet oxygen quenchers and traps for OH• radicals, stabilise proteins endogenously.

The high capability of proline to quench singlet oxygen and hydroxyl radicals can be well understood by its chemical properties.

Pyrrolidine, which forms the 5-membered ring of proline, has a remarkable low IP, and therefore proline is capable of forming a charge transfer complex and can quench singlet oxygen effectively. Proline reacts with OH• under hydrogen abstraction by forming the most stable radical, which carries the spin on the C-5 atom. Therefore, proline accumulation in high amounts in plants under stress could be well understood by its property to scavenge reactive oxygen species. Genetic engineering to enhance proline biosynthesis in important crop plants, which do not accumulate proline under stress, may be an important strategy to tackle the high level of reactive oxygen species generated during stressful conditions. Furthermore, dermatology may profit from understanding the action of proline-rich proteins in order to protect tissues from radiation-induced damages.

Examples

1. EPR on in-vitro systems

We used electron paramagnetic resonance (EPR) to demonstrate the excellent singlet-oxygen quenching properties of proline in solution with photo- sensitiser. Experiments were also performed with a purified small proline rich protein (SPRR4, 16.4% proline) and showed a quenching effect, which on a molar ratio was comparable to that of free proline. These experiments were repeated with different protein samples (various members of the SPRR family having up to 39.7% proline and other unrelated proteins with low proline content), in order to quantify the quenching properties of protein-bound proline. The synergistic effect of clustered proline residues in a protein is also studied.

Materials and methods:

Various human small proline rich protein (SPRR) coding sequences were introduced into expression vector pET-16 SK and the proteins were

overproduced in bacterial strain BL21(DE3)*(RP). Bacterial pellets were incubated in Na-citrate buffer pH 3.6, sonicated and centrifuged at 37,000 rpm in a Ti60 rotor (Beckman). Supernatants were applied to an FPLC column (Resource S, Pharmacia) and SPRR proteins were eluted at approximately 0.2 M NaCl, dialysed against 2000 volumes of Na - phosphate buffer pH 7.0 and stored at -80 °C. Proteins were >99% pure as could be judged after SDS-PAGE

In-vitro quenching of singlet oxygen by proline and SPRR was determined by EPR spectroscopy.

Results:

a) ROS quenching by proline

Time dependent increase in the level of singlet oxygen with various sensitizers was observed. Irrespective of the type of sensitiser used, presence of proline (20 mM) completely abolished the level of singlet oxygen. (fig.8)

Conclusion: These results clearly show that proline is very effective in scavenging singlet oxygen.

b) ROS quenching by SPRR

As shown in Fig. 9(A), a significant level of singlet oxygen was produced by photoirradiation of a sensitizer (toluidine blue) in the absence of added SPRR (control). The presence of SPRR4, SPRR3, SPRR2A or SPRR1B significantly reduced the level of singlet oxygen by 35%, 71%, 94% and 82% respectively (lower 3 spectra). The extent of quenching of singlet oxygen by different SPRRs was directly related to the number of proline residues in the protein (Fig. 9(B). SPRR2A which has 37.5% proline residues showed highest quenching ability while SPRR4 which contain 16.4 % proline residues showed lowest.

Conclusions: SPRRs have remarkably high singlet oxygen quenching ability. The quenching ability is directly related with the number of proline residues in the protein. (Fig. 11)

5

2. Cell survival under oxidative stress

Experiment 1:

Survival rates of various cell lines expressing high amounts of different SPRR proteins are determined after application of singlet-oxygen stress or other forms of stress. The protecting effect of varying proline concentrations in the culture medium is also analysed.

Materials and methods:

HeLa cells stably carrying the episomal vector pECV24 (Control), or vector containing SPRR1A (SPRR1), SPRR2E (SPRR2) or SPRR3 (SPRR3) gene constructs, were exposed to UVA or to singlet oxygen stress. UVA stress was induced by exposing the cells to total dose of 2000J/m^2 of UVA. Singlet oxygen stress was induced by treating the cells with different concentration of Ce6 (0 to 10 μM) and immediately exposing them to white light (1200μE $m^{-2}sec^{-1}$) for 10 min. Subsequent to UVA or singlet oxygen stress, cells were washed with PBS buffer and after adding fresh growth medium were incubated in dark at 37°C. After 48 hours, the survival of the cells was assayed by cell proliferation reagent WST-1 (Roche).

For evaluating the protective effect of free external proline on the survival of HeLa cells during exposure to singlet oxygen stress, free proline was added to the medium at concentrations ranging from 0 to 200 mM just prior to the stress. Singlet oxygen stress was induced by treating the cells with 2 μM Ce6 and immediately exposing them to white light (1200μE $m^{-2}sec^{-1}$) for 10 min. Subsequent to singlet oxygen stress, cells were washed with PBS buffer and after adding fresh growth medium, cells were

incubated in dark at 37°C. After 48 hours, the survival of the cells was assayed by cell proliferation reagent WST-1 (Roche).

Results:

As shown in Fig. 10 (A, left) UVA exposure significantly reduced the survival of HeLa cells carrying empty vector (control). On the other hand, HeLa cells expressing either SPRR1A, SPRR2E or SPRR3 show increased survival than control cells after UVA exposure. These results suggest that expression of SPRR has a significant protective effect against UVA stress.

Fig. 10 (A, right) shows the survival of HeLa cells, not expressing (control) or expressing either SPRR1A, SPRR2E or SPRR3, after exposing them to singlet oxygen stress provided by increasing concentrations of Ce6. The survival of the cells was reduced with increase in the concentration of Ce6. In general cells expressing SPRRs show enhanced survival than control cells. The protective effect of SPRRs expression was more striking at lower dose of Ce6 (2μM). Interestingly, the cells which give the greatest protection against UVA show also the highest resistance against Ce6, indicating that in both instance the same kind of damage is counteracted.

As shown in Fig. 10(B), the survival of HeLa cells during exposure to singlet oxygen stress was increased when free proline was added to the medium at concentrations ranging from 0 to 200 mM just prior to the stress.

Conclusion: Expression of SPRR proteins increases the resistance of cells against UVA or singlet oxygen stress. Presence of free proline in the medium also provided protection to the cells during singlet oxygen stress.

<u>Experiment 2</u>

In this experiment the ROS quenching ability of purified human epidermal cornified envelopes (CE) was analysed by direct measurement of the half-life of singlet oxygen in the presence or absence of intact or fractured CEs

Materials and Methods: The uppermost layer of our skin consist of dead cells which are encapsulated into a thick proteinaceous layer called cornified cell envelope (CE). Under normal conditions 5% of the total protein in CE are SPRRs. However, under UV exposure the amount of SPRRs significantly increased in the CE (Cabral et al, 2001). Consequently, CE's were isolated from peeled human (sun-burned) skin by boiling the skin pieces in buffer containing SDS and mercapto-ethanol. Under these conditions the cell contents are completely solubilized and only the cross-linked cornified envelopes are preserved and can be isolated. CE preparations were checked under the microscope for their purity.

Results:

The experiments shown in Fig. 11 demonstrate that isolated cornified cell envelope from UV irradiated skin which express high amount of SPRR proteins have an excellent singlet oxygen quenching ability. When intact cornified envelopes are broken by sonication, the SPRR protein rich layer is more exposed to the outside solvent. As a result the singlet oxygen quenching ability is further increased.

Conclusions:

These results clearly show that over-expression of SPRR proteins in skin upon exposure to UV irradiation is linked to their ability to protect against UV induced singlet oxygen production. Expression of SPPRs has a protective effect against UV irradiation in the outermost layer of our skin. This protective effect can be enhanced by use of external proline.

Furthermore, this experiment shows that ROS quenching is an inherent property of human cornified envelopes.

3. In vivo studies

Experiment 1

Effect of proline feeding on ageing in the double knockpout mouse($XPA^{-/-}CSB^{-/-}$)

In these experiments, double knockout mice ($XPA^{-/-}CSB^{-/-}$) (Murai et al., 2001) that have developmental defects including neurological abnormalities, accelerated apoptosis, mostly die during birth or at early postnatal age were used. Experiments were performed in collaboration with the group of Prof. Dr. J. Hoeijmakers (Dept. Genetics, EUR, Rotterdam)

Materials and methods:

Single knockout parents ($XPA^{+/-}CSB^{-/-}$ and $XPA^{-/-}CSB^{+/-}$) were crossed to generate double knockout pups ($XPA^{-/-}CSB^{-/-}$). Before mating as well as during whole pregnancy and lactation, the mother mouse was put on a proline diet. Proline was added to the drinking water (0.2M proline in water; 135-150 mg proline per day per mouse).

Results: Proline showed excellent protection *in utero* and the percentage of the born double knockouts pups increased from 9% (control mice) to at least 20% (in proline fed mice). In addition the percentage of overall survival of double knockout pups during the first 3 weeks after birth was higher in pups born from a proline fed mother, as compared to those born from a non-proline fed mother.

Other comments: The double knockout mouse model ($XPA^{-/-}CSB^{-/-}$) has been shown earlier to exhibit neurological abnormalities, postnatal growth

failure and premature death. Since in human, cerebral neurodegeneration of XPA and CS patients is suggested to be linked with oxidative stress (Hayashi et al., 2001), we used double knockout mouse model ($XPA^{-/-}CSB^{-/-}$) to study if proline can increase the survival of double knockout mouse by protecting against oxidative stress. Our results show clear protection of double knockout pups. ***in utero*** and during early postnatal age.

These results also suggest that proline can be useful in treatment of oxidative damage linked neurodegenerative diseases. Giasson et al (2000) have shown that oxidative damage is directly linked to neurodegenerative synucleinopathies, particularly Parkinson's.

Experiment 2

In these experiments hairless mice (SKH-1) were used to demonstrate that proline provides excellent protection against ROS induced skin damage in these mice. ROS mediated skin damage was selectively induced by topical application of ALA (5-aminolevulinic acid). ALA application leads to intracellular accumulation of protoporphyrin IX (PpIX). PpIX is a sensitizer which in presence of light and oxygen generates ROS and causes visual skin damage (e.g. localised tissue necrosis).

Materials and methods: Five weeks old hairless mice (SKH-1) were kept on proline diet. Proline was added to the drinking water (0.2M proline in water; 135-150 mg proline per day per mouse). Control mice were fed with only water (without proline). After taking proline or non-proline water for three days, the dorsal skin of the mice was smeared with cream (Lanett) containing 20% ALA They were incubated in dark for four hours and subsequently were exposed to light (1,6 KJ/m2) provided from TL-03 lamp (Philips). Afterward the mice were kept in dark. Visual skin damage was monitored 24 h and 48 h after the light treatment. These

experiments were performed in collaboration with the group of Dr. F. de Gruijl (Dept. Dermatology, Leiden University).

Results: As can be seen in Fig. 12, the control mice showed visual skin damage at the location where ALA was applied. However, proline fed mice do not show any skin damage at the place where ALA was applied.

Experiment 3

In these experiments hairless mice (SKH-1) were used to demonstrate that proline reduce light sensitivity of mice after photodynamic therapy. Photodynamic therapy (PDT) involves the administration of a photosensitizer (or precursor of sensitizer which gets converted into sensitizer inside the body) which preferentially get localised in tumour tissue and upon illumination results in photodamage and subsequent elemination of tumour. One of the major problems in PDT is that the photosensitizer remains in the body for quite some time and patients remain sensitive to light for many days or weeks after the PDT. We demonstrate here that taking proline orally can reduce the sensitivity of mice to light after PDT.

Materials and methods: Hairless mice (SKH-1) were exposed to photodynamic therapy by applying 20% ALA in cream. They were covered with tape and small areas were exposed to light to induce localised skin photodamage. Subsequently the tape was removed and mice were kept in normal light. Half of them (6 mice out of 12) were fed with proline (150 mg/day per mouse). The visual skin damage was assessed with respect to erythema and/or edema.

Results: Mice which were fed with proline remained normal and did not show any skin damage. However mice which were not fed with proline show erythema and/or edema at many areas on the skin.

Table: Sensitivity of mice to light after PDT

Time after PDT	Control mice Erythema/Edema	Proline fed mice Erythema/Edema
24 h	+ / -	-
48 h	++ /+	-
72h	+++ /++	-
96 h	++ /++	-

Experiment 4

Study on effect of proline on human skin after UVB irradiation

Volunteers were exposed to different doses of UVB irradiation (provided by a mercury lamp) on both arms. Subsequently cream alone or cream with proline (10 mM) was applied. Erythema was monitored after 8, 16 and 24 hours.

Results: Proline provided protection to the skin against UVB exposure. Erythema of the skin monitored 8, 16 and 24 hours after exposure to different doses of UVB (2700 J/m2, 3600 J/m2) was lower in the area of the skin where cream containing proline was applied compared to the area where only cream without proline was applied.

FIGURE LEGENDS:

Fig. 1:A: Structure of L-proline, B: Structure of hydroxyproline.

Fig. 2: Proline biosynthesis and oxidation showing ring opening from proline to glutamate and proline-glutamate electron sponge system

Fig. 3: Structure and life time (τ) of electronically excited oxygen molecules. The $^1\Sigma g^-$ ground state is a triplet. The two singlet states $^1\Delta g$ and $^1\Sigma g^+$ are electronically excited. (Adapted from W. Adam (1981) Chem. Unsere Zeit 15, 190-196)

Fig. 4: Oxidation level of reactive oxygen species.

Fig. 5: Photochemical production of singlet oxygen ($^3O_2$) by a photosensitiser (D) and its reaction with amines (A).

Fig. 6: Ionisation potentials of some amines measure in the gas phase.

Fig. 7: Proline residues in polypeptide/proteins. The prolyl residues are selectively oxidised by OH° to glutamic acid.

Fig. 8: *Effect of proline on singlet oxygen production.* Singlet oxygen ($^1O_2$) were generated through photoirradiation of sensitiser (toluidine blue, fluorescein, rose bengal or hematoporphyrin) and were detected by the formation of TEMPO due to reaction of TEMP with $^1O_2$. The sample contained 1 mM sensitiser and 10 mM TEMP was irradiated to white light ($1200\mu E\ m^{-2}sec^{-1}$) for various times in the absence (-o-) or presence of 20 mM proline (-•-) and EPR spectra was recorded with X-band EPR spectrometer. Results are average of 3 independent experiments

Fig. 9: *Effect of small proline rich proteins (SPRR) on singlet oxygen quenching.* Singlet oxygen ($^1O_2$) were generated through photoirradiation of sensitiser (toluidine blue) and were detected by the formation of TEMPO due to reaction of TEMP with $^1O_2$. The sample containing 1 mM sensitiser and 10 mM TEMP was irradiated with white light (1200μE $m^{-2}sec^{-1}$) for 10 minutes in the absence (control) or presence of 0.35 μM SPRRs and EPR spectra was recorded with an X-band EPR spectrometer. (A) EPR spectra; (B) relationship between proline content in SPRR and $^1O_2$ quenching.

Fig. 10. (A) Protective effect of SPRR expression on the survival of human HeLa cells after exposure to UVA stress (left), or direct singlet oxygen stress (right). HeLa cells carrying the episomal vector PECV24 (Control), or vector containing SPRR1A (SPRR1), SPRR2E (SPRR2) or SPRR3 (SPRR3) gene constructs, were exposed to UVA (2000J/m^2) or to different concentrations of $^1O_2$ generating sensitizer, Ce6 in the presence of white light (1200μE $m^{-2}sec^{-1}$). Survival of the cells was monitored 48 h after the stress by using the WST-1 assay (ROCHE).
(B) Protective effect of different concentrations of external proline on the survival of HeLa cells during exposure to $^1O_2$ stress induced by 2 μM Ce6 in presence of white light (1200μE $m^{-2}sec^{-1}$).

Fig. 11. Singlet oxygen quenching ability of cornified cell envelope isolated from UV irradiated human skin. (A) intact CE and sonicated CE (B) Time resolved detection of singlet oxygen quenching in D_2O (—), intact CE (---), and sonicated CE (...).

Fig. 12. ROS induced skin damage in mice and its prevention by proline. White arrows show the location where ALA was topically applied. Control mice (A); Proline fed mice (B). C and D are zoom view of A and B respectively.

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Claims

1. Use of proline and/or a functional equivalent thereof for quenching ROS and/or radicals.
2. Use of proline according to claim1, wherein at least part of the proline is provided as protein and/or peptide comprising at least 8 mol% proline and/or a functional equivalent thereof.
3. Use of a proline according to claim 1 or 2 wherein said proline is provided in a composition comprising at least 0.01 millimolar proline.
4. Use of a proline according to claim 2 or 3 wherein said protein is provided in a composition comprising at least 0.01 micromolar protein.
5. Use of proline and/or a functional equivalent thereof for preventing and/or diminishing damage by ROS and/or radicals.
6. Use of proline and/or a functional equivalent thereof for the preparation of a medicament for diminishing or preventing damage to cells and/or tissues by ROS and/or radicals.
7. Use according to any one of claims 1-6 wherein said proline and/or a functional equivalent thereof is added to a food substance.
8. Use according to any one of claims 1-6 wherein said proline and/or a functional equivalent thereof is added to a topical composition.
9. Use of proline and/or a functional equivalent thereof as an active substance in the preparation of a pharmaceutical composition.
10. A topical composition comprising proline and/or a equivalent thereof and a suitable carrier or diluent, for diminishing or preventing damage to cells and/or tissues by ROS and/or radicals.
11. A topical composition according to claim 10 wherein said proline is at least in a concentration of 0.01 millimolar.

12. A topical composition according to claim 10 or 11 wherein at least part of said proline is provided as protein and/or peptide comprising at least 8 mol% proline and/or a functional equivalent thereof.
13. A sun-tanning product comprising a topical composition according to any of claims 10-12.
14. A cosmetic product comprising a topical composition according to any of claims 10-12 and/or a sun tanning product according to claim 12.
15. An oral sun-tanning product comprising a ROS and/or radicals quenching substance.
16. An oral sun-tanning product comprising a ROS and/or radicals quenching substance, which accumulates in skin.
17. An oral sun-tanning product according to claim15 or 16, comprising as an active substance proline and/or a functional equivalent thereof.
18. Use of a composition according to any of claims 10-17 for at least in part preventing ageing and/or decay of the treated tissue.
19. A method to prevent excessive tanning comprising applying to at least part of the skin a composition according to claim 13, prior to exposure of the body of a subject to a ROS and/or radicals inducing condition.
20. A method to prevent excessive tanning comprising administering to a subject a composition according to any of claims 10-17, prior to exposure of the body of a subject to a ROS and/or radicals inducing condition.
21. A method to treat inflammation characterized in that proline or a functional equivalent is used as an active substance
22. A method to treat local inflammation in the skin comprising applying to at least part of the skin of a subject a composition according to any of claims 10-12.

23. A method to treat sunburn in the skin comprising applying to at least part of the skin of a subject a composition according to any of claims 10-14.

24. An after-sun product comprising a ROS and/or radicals quenching substance.

25. An after-sun product comprising a topical composition according to any of claims 10-12.

26. An oral after-sun product comprising a ROS and/or radicals quenching substance, which accumulates in skin.

27. An oral after-sun product according to claim 26, comprising as an active substance proline and/or a functional equivalent thereof.

28. A method to protect hair, eyes, nails, skin and teeth against damage by ROS and/or radicals comprising applying a composition according to any of claims 10-12, prior to, and/or during, and/or after exposure of the body to a ROS and/or radical inducing condition.

29. A pharmaceutical composition comprising as an active substance proline and/or a functional equivalent thereof and a suitable carrier or diluent.

30. Use according to claim 6 or 9 for the preparation of a medicament for the treatment of ROS and/or radicals -related disease comprising damaging radiation induced skin damage, inflammatory skin diseases, fibrosis, skin cancer, psoriasis, periodontal disease, pathological conditions of the stomach and gut affecting mucosal barrier function, pathologies of lung, oral and nasal epithelium due to cigarette smoke, photo-aging of the skin, wound healing, aging and age related diseases, cardiovascular diseases, artherosclerosis, Alzheimer's disease and neurodegenerative diseases, dementia, Parkinson's disease, inflammatory airway disorders, male infertility, and/or breast cancer.

31. Use according to claim 6 or 9 for the preparation of a medicament for diminishing or preventing damaging effects by ROS and/or

radicals in a subject and/or an animal exposed to high doses of damaging and/or ionising and/or cosmic rays.

32. A method to protect cells and tissues of a subject or an animal against damage by ROS and/or radicals by administration of a pharmaceutical composition according to claim 29 prior to, and/or during, and/or after a ROS and/or radicals inducing treatment or diagnosis.

33. The use of proline and/or a functional equivalent for the preparation of a food anti-oxidant.

34. The use of proline and/or a functional equivalent for the preparation of a nutraceutical composition.

35. A neutraceutical composition comprising as an active substance proline and/or a functional equivalent thereof, for the protection of mammals against oxidative damage.

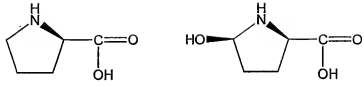
36. A neutraceutical composition according to claim35, wherein said proline is at least in a concentration of 0.01 millimolar

37. A neutraceutical composition according to claim 35 or 36 wherein at least part of said proline is provided as protein and/or peptide comprising at least 8 mol% proline and/or a functional equivalent thereof.

38. A method to protect mammals against oxidative damage comprising increasing the proline level of their food and drink to at least 0.01 millimolar by adding proline and/or a functional equivalent.

39. A method to diagnose ROS and/or radical -related damage and/or disease comprising measuring the level of proline or a functional equivalent thereof in a sample of damaged or diseased tissue.

40. A method to diagnose ROS-related damage and/or disease comprising measuring the expression levels of the PrP, SPRR and LEP gene families.



A B

Fig. 1

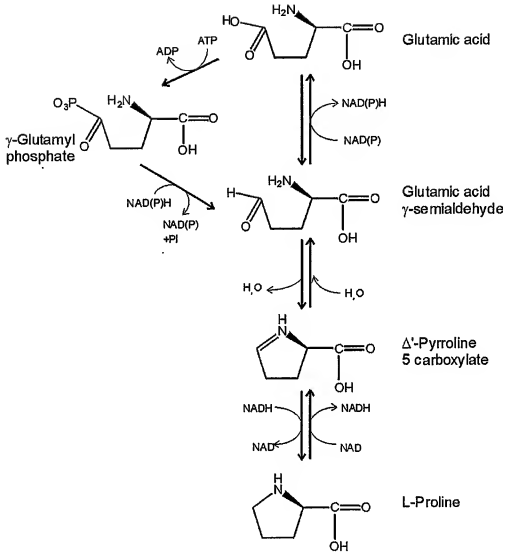

Glutamic acid
ATP
ADP
NAD(P)H
NAD(P)
γ-Glutamyl phosphate
NAD(P)H
NAD(P)
+PI
Glutamic acid γ-semialdehyde
H2O
H2O
Δ'-Pyrroline 5 carboxylate
NADH
NADH
NAD
NAD
L-Proline

Fig. 2

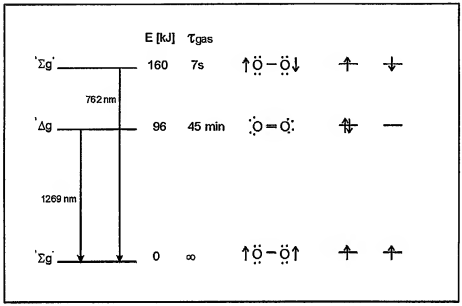


Fig. 3

oxygen	O_2	$+ e^-, H^+$
superoxide	$HO_2^\bullet$	$+ e^-, H^+$
peroxide	H_2O_2	
hydroxy	$2\ OH^\bullet$	$+ e^-, H^+$
hydroxy/hydroxide	$OH^\bullet + OH^-$	$+ e^-, H^+$
water	$2\ H_2O$	

Fig. 4

$^1D + h\nu \rightarrow {}^1D^*$	(photochemical excitation)	[1]
$^1D^* \rightarrow {}^3D$	(inter-system crossing)	[2]
$^3D + {}^3O_2 \rightarrow {}^1D + {}^1O_2$	(photo-sensitization)	[3]
$^1O_2 + A \square {}^1[O_2^{\delta-} \leftarrow A^{\delta+}]$	(reversible formation of a charge-transfer complex)	[4]
$^1[O_2^{\delta-} \leftarrow A^{\delta+}] \rightarrow {}^3[O_2^{\delta-} \leftarrow A^{\delta+}] \rightarrow {}^3O_2 + A$	(physical quenching)	[5]
$^1[O_2^{\delta-} \leftarrow A^{\delta+}] \rightarrow$ products	(chemical quenching)	[6]

Fig. 5

Primary amine:	Ethyl amine $CH_3CH_2NH_2$	8.9 eV
Secondary amine:	Dimethyl amine $(CH_3)_2HNH_2$	8.2 eV
Pyrrolidine:		8.0 eV
Tertiary amine:	Trimethyl amine $N(CH_3)_3$	7.8 eV

Fig. 6

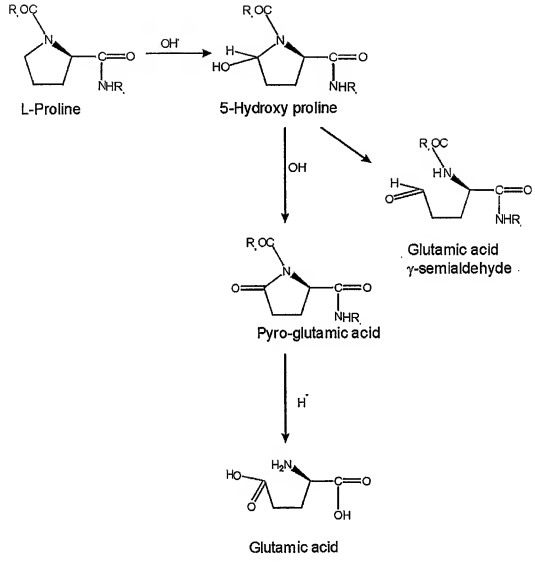

L-Proline
5-Hydroxy proline
Glutamic acid
γ-semialdehyde
Pyro-glutamic acid
Glutamic acid

Fig. 7

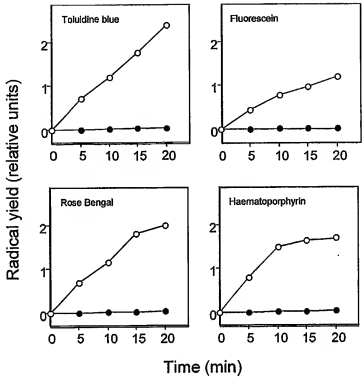

Toluidine blue
Fluorescein
Rose Bengal
Haematoporphyrin
Radical yield (relative units)
Time (min)
0
1
2
0 5 10 15 20

Fig. 8

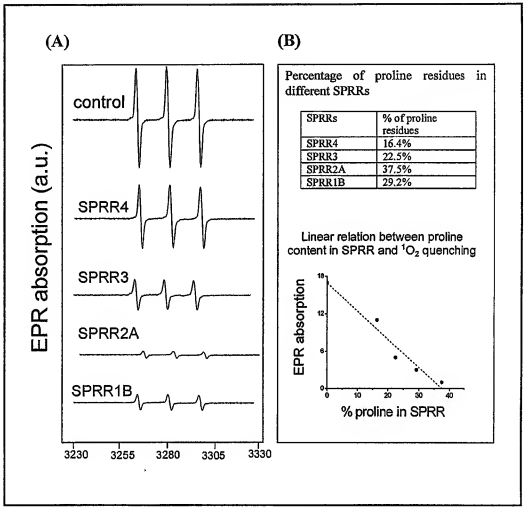

(A)
(B)
EPR absorption (a.u.)
control
SPRR4
SPRR3
SPRR2A
SPRR1B
3230
3255
3280
3305
3330
Percentage of proline residues in different SPRRs
SPRRs
% of proline residues
SPRR4
16.4%
SPRR3
22.5%
SPRR2A
37.5%
SPRR1B
29.2%
Linear relation between proline content in SPRR and $^1O_2$ quenching
EPR absorption
% proline in SPRR

Fig. 9

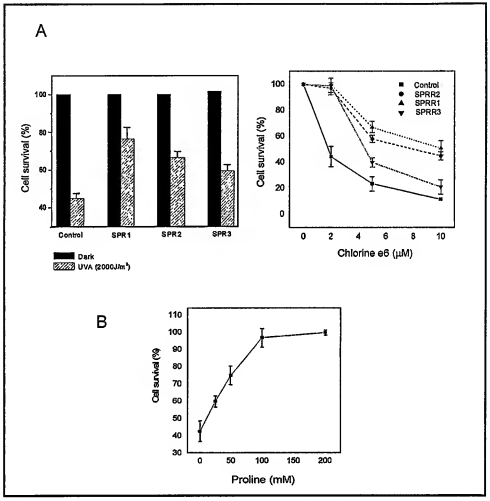
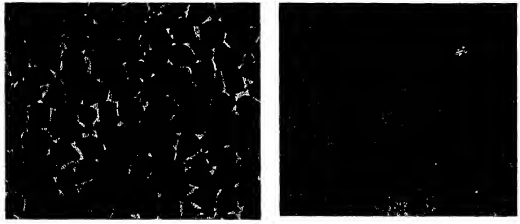

A
Cell survival (%)
100
80
60
40
Control
SPR1
SPR2
SPR3
Dark
UVA (2000Jm-2)
Cell survival (%)
100
80
60
40
20
0
Control
SPRR2
SPRR1
SPRR3
0
2
4
6
8
10
Chlorine e6 (μM)
B
Cell survival (%)
110
100
90
80
70
60
50
40
30
0
50
100
150
200
Proline (mM)

Fig. 10

(A)



(B)

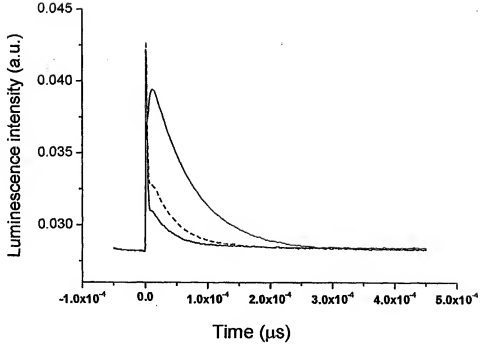

0.045
0.040
0.035
0.030
Luminescence intensity (a.u.)
-1.0x10⁻⁴
0.0
1.0x10⁻⁴
2.0x10⁻⁴
3.0x10⁻⁴
4.0x10⁻⁴
5.0x10⁻⁴
Time (μs)

Fig. 11

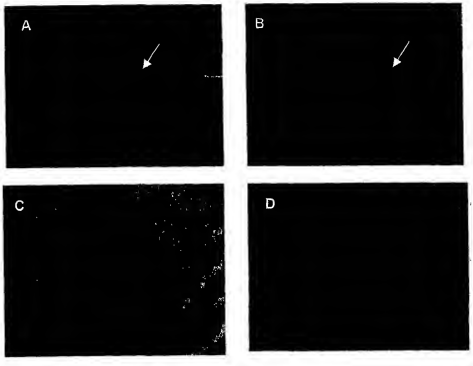


Fig. 12